INDUCTION OF IMMUNOLOGICAL TOLERANCE BY INTRA-COELOMIC GRAFTS IN THE 4-DAY CHICK EMBRYO

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When cells or antigens are injected into an embryo or a newborn animal a condition of tolerance to the foreign stimulus may be induced. Although the mechanism involved in this induction is not well known, we may entertain two possibilities: (1) that the embryo may react with the cells or antigen, thereby revealing the development of a certain level of immunological competence, and manifest either a tolerant or an immune reaction depending on the dosage (Howard and Michie, 1962; Michie and Howard, 1962). However (2) the host embryo may also nourish the proliferation of the foreign cells and permit the establishment of a chimeric condition which is frequently obtained in tolerant animals (Billingham *et al.,* 1952; Hasek and Hort, 1960; Stone *et al.,* 1965). Although the mechanism is not clear, we may presume that the requirement for immunological competence is not involved in such cases.

In the chick embryo, it is possible to examine these two alternatives as well as elucidate the role of competence in the induction of tolerance by implanting cells or antigens into the coelom of 4-day embryos, well before the onset of competence. Immunologically competent cells, as measured by their ability to elicit a splenomegaly, are not detected until immediately after hatching (Solomon, 1961; Mun *et al.,* 1962). Solomon (1963) reported the sensitization of host lymphocytes as measured by a depressed splenomegaly in the host during the eleventh to seventeenth days of incubation. Ackerman and Knouff (1964) were able to identify certain cell types which may be associated with the production of antibody in the thymus of older 10- and 14-day chick embryos.

We ask first: Can tolerance be induced in the 4-day chick embryo? If so, we may next inquire: Would a greater degree of tolerance be obtained by the exchange of cells or tissue from the same stage of development or by tissue from embryos older than 10 days which may contain immunologically competent cells (Mun, 1965)?

Materials and Methods

Two series of experiments were conducted, one at Orono, Maine, and the other at East Lansing, Michigan. In the first series of experiments, the donor tissues were obtained from a White Leghorn (WL) strain obtained from SPAFAS, Inc., Norwich, Conn., which has maintained for 10 or more years a closed flock with continuous inbreeding, but not necessarily with brother-sister mating. The hosts were derived from a cross between a Rhode Island Red male and a Barred Rock

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female (BR × RIR). In the second series of experiments, donor tissues were obtained from Line 7 embryos and hosts were derived from a cross between Line 15 I and Line 6. These lines have been maintained as independent inbred lines at the Regional Poultry Research Laboratory since 1939 (Crittenden *et al.,* 1964).

The intracoelomic grafting technique has been previously described by Hamburger (1960) and Dossel (1954). The eggs were incubated for 86 to 96 hours at 99° F. and 85% relative humidity. Embryos which had attained normal developmental stage 21 (Hamburger and Hamilton, 1951) with the allantois almost in contact with the head were selected. A cut was made with a steel needle through both the vitelline membrane and the somatopleure, in the small space between the allantois and the head. The donor tissue, approximately 0.1 mm.^{3}, was then pushed through this opening and into the coelom toward the base of the allantois with a curved and blunted glass needle. The operated eggs with their pointed ends down were placed in the incubator and permitted to hatch.

TABLE I

Mean survival time of homografts in untreated hosts

Host	Donor	No. cases	MST* days	Standard deviation	Range of survival time (days)
BR × RIR	WL (SPAFAS)	53	8.1 ± 0.5	1.89	5–14
151 × 6	Line 7	40	16.8 ± 2.0	6.29	6–31

* Plus and minus 95% confidence limits.

Two to 4 days after hatching, the chicks were divided into groups of four to six, each group being made up of both operated, and unoperated or sham-operated chicks. Each chick in the group then received a skin graft from a donor of the same age and of the same strain as the previous embryonic donor. The donor chick was discarded. The skin grafting technique developed by Cannon and Longmire (1952) was employed. The chicks were randomly numbered and not identified according to treatment. The grafts were read at one- or two-day intervals for the first two to three weeks post-operation and later at weekly intervals. The condition of the grafts in operated animals was compared with autografts as well as grafts in unoperated controls, and the time at which the first signs of rejection appeared was noted. Rejection was usually marked by a sudden darkening of the graft, *e.g.,* from pink or yellow to dark purple or brown, as well as a change in the surface texture, *e.g.,* from a soft, pliable condition to a smooth, hard surface (Polley *et al.,* 1960). After this stage, the hard scab which is formed eventually drops off and the resultant bare area may persist for a variable period of time, until the reappearance of host feathers oriented in the normal direction.

RESULTS

When skin grafts were exchanged between 2- to 4-day hatched chicks from the same WL (SPAFAS) strain, eight out of sixteen grafts (50%) took successfully and remained for more than 20 days. On the other hand, skin grafts from WL (SPAFAS) donors on BR × RIR hosts were all rejected within 15 days (Table 1).

TABLE II

Survival of skin grafts in BR × RIR hosts following intracoelomic grafts of various tissues from WL (SPAFAS) embryos at different stages of development

Treatment	Total no. of cases	No. of grafts surviving 1 to 20 days	No. of grafts surviving more than 20 days
4-day pharyngeal pouch, limb bud or lens	16	16	0
7- or 8-day spleen	25	25	0
12-day spleen	7	7	0
15-day lens	5	5	0
14- to 21-day spleen or thymus	80	71	9 (11%)
None, or sham-operated	53	53	0

Fifteen experiments were conducted in which various tissues from WL (SPAFAS) were implanted into the coelom of BR × RIR embryos. Pooled data from these experiments show that limb buds, lens, or tissues from the region of the pharyngeal pouches 3 and 4 of 4-day embryos, spleen from 7-, 8-, or 12-day embryos and lens from 15-day embryos were not able to induce tolerance in BR × RIR hosts. However, tolerance was induced by spleen and thymus tissues from older 14- to 21-day embryos in 11% of the cases (Table II). Because no striking differences in ability of these two tissues to induce tolerance were observed in these preliminary studies, the data were pooled.

Because of the small percentage of treated animals manifesting tolerance, these experiments were repeated at East Lansing, Michigan, where embryos from highly inbred lines were available. Twenty-two out of 29 (75%) skin grafts between 2- to 4-day Line 7 chicks took successfully and remained more than 50 days. Although the homogeneity with respect to the histocompatibility loci in this particular line is not yet complete (Crittenden *et al.*, 1964), it is greater than that in the WL (SPAFAS) strain (50%). Skin grafts from hatched chicks of Line 7 placed on sham- or saline-operated or unoperated chicks of Lines 15 I × 6 were all rejected within 32 days (Table I).

Ten experiments were conducted in which donor tissues from Line 7 embryos were implanted in the coelom of 4-day 15 I × 6 embryos. Donor tissues were

TABLE III

Survival of skin grafts in 15 I × 6 hosts following intracoelomic grafts of various tissues from Line 7 embryos at different stages of development

Treatment	Total no. of cases	Graft survival time in days		
		1 to 32 days	33 to 50 days	More than 50 days
Intracoelomic grafts of				
(1) 4-day embryonic limb, liver, pouches 3 and 4	64	52	4	8(12.5%)
(2) 9- to 18-day embryonic spleen, thymus, and liver	44	16	10	18(41%)
(3) 1-day hatched chick spleen, thymus	16	10	4	2
Control or sham	40	40	0	0
Autograft 15 I × 6	39	1	0	38(97%)

obtained from limb buds, third and fourth pharyngeal pouches, and liver of 4-day embryos. Spleen, liver and thymus tissues were obtained from 9-day embryos to 1-day hatched chicks. Table III shows that a significantly greater proportion of skin grafts lasting more than 50 days was obtained in chicks receiving intracoelomic grafts from older (9- to 18-day) embryos than from younger (4-day) embryos ($P < 0.005$). Runts disease was observed in a few cases receiving intracoelomic grafts from 1-day hatched chick tissues.

Discussion

Tolerance can be induced in the chick embryo by joining their chorioallantoic membranes on or about the 10th day of incubation (Hasek, Hraba and Hort, 1958) or by cross-transfusion of blood on the 10th to 16th days of incubation (Terasaki, Cannon and Longmire, 1958).

The present data show clearly that tolerance can also be induced in the 4-day chick embryo by implanting various tissues into the coelom. If the initial steps in the mechanism of tolerance induction involve the interaction of the foreign antigen with immunologically competent cells, we may conclude that competent cells are present in the chick embryo at this very early stage of development. However, because immunologically competent cells as measured by other means are not detected until at least after the 10th day of incubation, we may suggest that the foreign donor tissues persist in the host environment and later react with competent host cells as they appear (Mun *et al.*, 1962). On the other hand, the observation that tolerance induction was enhanced by older, more differentiated tissue argues against the notion that tolerance is solely the result of mutual exchange, or persistence of donor tissue or cells in the host environment, and compels us to consider the immediate impact of the foreign cells on the host environment.

There may be several explanations to account for the difference in the ability to induce tolerance:

(1) The older tissues "took" better than grafts from younger donors. Volpe and Gebhardt (1965) observed in the frog that larger homografts, comprising two complete lateral neural folds, survived and persisted indefinitely, while smaller single lateral neural fold homografts were almost invariably eventually rejected. Thus, the older donor grafts with a greater amount of antigen may demonstrate a larger percentage of tolerant cases mainly because of their greater ability to survive in the embryonic environment.

(2) On the other hand if we may assume that both older and younger grafts take equally well, the greater ability of the older tissue to induce tolerance may likewise be due to the amount of antigen. Howard and Michie (1962), Michie and Howard (1962) and others have shown that a larger dose would result in tolerance but a smaller dose of the same antigen would elicit sensitivity. However, we found that intracoelomic grafting of larger pieces of tissues, almost two to three times the usual size (0.1 to 0.3 $mm.^3$), from either older or younger donors, did not result in the induction of a greater degree of tolerance. The use of large pieces of lens tissue from 15-day chick embryos also did not induce tolerance.

(3) The enhancement of tolerance by the grafts from older donors may also be due to qualitative differences, as well as quantitative differences in antigen supply (Billingham and Silvers, 1962). Ebert (1951) discovered the appearance of

a spleen specific antigen on or about the 18th day of incubation. However, studies on the development of the B blood antigens which are strongly associated with histocompatibility in the chick reveal that they can be detected as early as the 7th day of incubation (L. W. Johnson and W. E. Briles, personal communication).

(4) This leads us to consider another possibility: the impact of immunologically competent cells, which we may find in the 14- to 21-day donor, on the host environment. Jensen and Simonsen (1962) have observed in parabiosis experiments in highly inbred mice, a facilitation of tolerance by the same antigenic stimulus when the parabiont to become tolerant was exposed to a graft-vs.-host reaction from its partner at the same time. The immunologically competent cells may respond to the host antigen by proliferation and the release of greater amounts of donor antigen, thus increasing their effective dosage very rapidly (Billingham and Silvers, 1961, p. 127; see discussion by Burch and Burwell, 1965, p. 271). In the chick, the immunologically competent donor cells may also act to stimulate proliferation of the embryonic host spleen cells contributing to the observed organ enlargement (Danchakoff, 1916; Biggs and Payne, 1961; DeLanney *et al.,* 1962; Mun and Burns, 1965). The role of these host-donor cell interactions in the mechanism of tolerance induction remains to be explored.

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Summary

1. In a series of experiments in which non-inbred material was used, 9 out of 80 skin grafts from a White Leghorn strain survived more than 20 days on Barred Rock × Rhode Island Red hosts which had received intracoelomic grafts of spleen and thymus from older (14- to 21-day) embryos of the same donor strain. Hosts which had received intracoelomic grafts of pharyngeal pouches 3 and 4, limb buds and lens from 4-day embryos, or spleens from 7-, 8-, or 12-day embryos or lens tissue from 15-day embryos, rejected skin grafts from the same donor strain within 20 days.

2. When highly inbred material was used, tolerance was induced in Line 15 I × 6 hosts by intracoelomic grafts of limb buds, liver, or pouches 3 and 4 from Line 7 embryos of 4 days. However, a significantly greater degree of tolerance was induced by spleen and thymus tissues from older 9- to 18-day embryos of the same donor strain. The possible impact of near-immunologically competent cells on host cells in the induction of tolerance was considered.

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